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PRINCIPAL INVESTIGATOR: Kevin Strange, PhD

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Kevin Strange, Ph.D.; Viravuth Yin, Ph.D.

E-Mail: kstrange@mdibl.org; vyin@mdibl.org

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Mount Desert Island Biological Laboratory
Old Bar Harbor Road
PO Box 35
Salisbury Cove, ME 04672

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14. ABSTRACT
The inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million affected U. S. citizens and active service members. Development of in vivo therapies that restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. Thus, characterizing ancestral regulatory circuitry controlling regeneration is a necessary and direct route to identifying the mechanistic causes of regenerative failure in mammals. This proposal offers unique promise in guiding the targeted development of in vivo therapies to restore/augment human limb regeneration. We will leverage our recent discovery that regenerative ability is widespread in basal vertebrates to conduct the first comparative analysis of appendage regeneration that incorporates model systems from all major groups of limbed vertebrates- cartilaginous fishes, ray-finned fishes, and tetrapods. Our unique approach will identify novel functional requirements for genes/gene networks in regulating appendage regeneration by marrying a comparative organismal approach with state-of-the-art systems-level analyses of gene expression using next generation RNA sequencing and functional analysis of candidate regulators in the genetically tractable zebrafish model system through in vivo disruption of gene function.

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Table of Contents

THE CENTER FOR REGENERATIVE BIOLOGY AND MEDICINE AT MOUNT DESERT ISLAND BIOLOGICAL LABORATORY

INTRODUCTION:

The inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million affected U. S. citizens and active service members. Development of *in vivo* therapies that restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. Thus, characterizing ancestral regulatory circuitry controlling regeneration is a necessary and direct route to identifying the mechanistic causes of regenerative failure in mammals. This proposal offers unique promise in guiding the targeted development of *in vivo* therapies to restore/augment human limb regeneration. We will leverage our recent discovery that regenerative ability is widespread in basal vertebrates to conduct the first comparative analysis of appendage regeneration that incorporates model systems from all major groups of limbed vertebrates- cartilaginous fishes, ray-finned fishes, and tetrapods. Our unique approach will identify novel functional requirements for genes/gene networks in regulating appendage regeneration by marrying a comparative organismal approach with state-of-the-art systems-level analyses of gene expression using next generation RNA sequencing and functional analysis of candidate regulators in the genetically tractable zebrafish model system through *in vivo* disruption of gene function.

BODY:

Specific Aim 1: Characterize mRNA expression profiles in regenerating salamander (*Ambystoma mexicanum*) limbs and *Polypterus senegalus* fins. This work will be accomplished by **a**) collecting tissue and mRNA from regenerating salamander and *Polypterus* appendages, **b**) characterizing gene expression profiles by DNA sequencing using Solexa/Illumina technology, **c**) assembling and annotating transcriptome sequence, **d**) identifying conserved regulators of limb/fin regeneration by quantitative bioinformatics analysis, and **e**) assessing functional requirements of candidate regulators.

Research Accomplishments: The major focus for Aim 1 during Year 2 was to analyze the limb regeneration deep sequencing dataset focused exclusively on mRNA transcripts. We reasoned that mRNAs that are important for the regenerative response are likely to be differentially regulated in response to injury. Thus, for each species, we compared and contrasted the dataset between uninjured and 7 days post-amputation (dpa) samples, a time of active regenerative growth. To further refine the genes important for regeneration, we applied another selection factor to identify those genes that are similarly controlled in both *Polypterus* and axolotl samples. These comparisons revealed a total of 2779 shared genes that are significantly upregulated during regeneration. Conversely, our analysis showed that 1082 genes are downregulated in response to limb amputation (Figure 1).

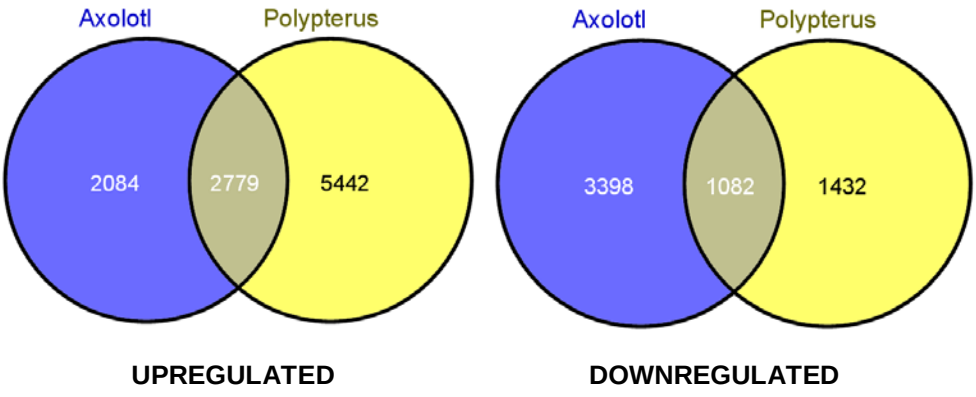

Figure 1: Venn diagram of UniProt protein sequence IDs among Axolotl and Polypterus contigs that were up-regulated and down regulated greater than 2-fold between 0 and 7 dpa. (Left) The common set of 2,779 upregulated UniProt protein sequence IDs were mapped to 2,617 human Ensembl genes. (Right) The common set of 1,082 downregulated UniProt protein sequence IDs were mapped to 1,019 human Ensembl genes.

From this dataset, we identified the 10 most highly upregulated genes during regeneration in both *Polypterus* and axolotl limb regeneration (Table 1). This collection of genes is involved in remodeling of the extracellular matrix (ECM), a process that is critical during cell movement and cellular dedifferentiation.

Table 1. Top ten most highly upregulated genes during axolotl and *Polypterus* limb regeneration.

Associated Gene	Description
KRT19	keratin 19 [Source:HGNC Symbol;Acc:6436]
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) [Source:HGNC Symbol;Acc:7176]
CTSK	cathepsin K [Source:HGNC Symbol;Acc:2536]
MMP13	matrix metallopeptidase 13 (collagenase 3) [Source:HGNC Symbol;Acc:7159]
KRT17	keratin 17 [Source:HGNC Symbol;Acc:6427]
MMP10	matrix metallopeptidase 10 (stromelysin 2) [Source:HGNC Symbol;Acc:7156]
PRDM1	PR domain containing 1, with ZNF domain [Source:HGNC Symbol;Acc:9346]
MMP1	matrix metallopeptidase 1 (interstitial collagenase) [Source:HGNC Symbol;Acc:7155]
NCF2	neutrophil cytosolic factor 2 [Source:HGNC Symbol;Acc:7661]
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase) [Source:HGNC Symbol;Acc:7173]

Our initial approach was to define functional contributions for *mmp9* due to the available specific pharmacological inhibitors. Initially, have focused on identifying an efficient delivery mechanism of the drug and an appropriate dose of AG3340 (MMP9 selective inhibitor) for axolotl and *Polypterus* regeneration studies. Using intraperitoneal microinjections at a concentration of 20mg/ml, we observed profound defects on limb and appendage regeneration. Unfortunately, during the course of our preliminary study we were informed that AG3340 would no longer be commercially available. Despite having tested other *mmp9* inhibitors, including inhibitory cyclic peptides, we were unable to identify a reagent that specifically and strongly impacts limb regeneration.

Despite these obstacles, we remain confident that genetic factors that remodel the ECM are important for promoting cellular reprogramming for tissue regeneration. Therefore, we will employ antisense technology to specifically reduce *mmp9* function in the axolotl and *Polypterus*. Antisense locked-nucleic acid (LNA) oligonucleotides specifically bind and degrade the target mRNA in an RNAse H dependent mechanism. Previously, we have employed this strategy to successfully deplete small noncoding RNAs. Once we confirm the efficacy of this reagent on *mmp9*, we will examine the impact on limb regeneration in greater detail. With this approach, we will also be able to perform simultaneous knockdown of multiple genes to elucidate potential combinatorial roles.

Specific Aim 2: Characterize microRNA (miRNA) expression profiles in regenerating salamander (*Ambystoma mexicanum*) limbs and *Polypterus senegalus* fins. This work will be accomplished by **a**) collecting tissues and isolating small RNA from regenerating salamander and *Polypterus* appendages, **b**) characterizing miRNA expression profiles generated by DNA sequencing using Solexa/Illumina technology, **c**) annotating miRNAs and characterizing their expression profiles in limb/fin regeneration, **d**) predicting miRNA targets and correlating miRNA expression with predicted targets, and **e**) assessing functional roles of candidate miRNAs.

Research Accomplishments: Gene regulatory networks are controlled at multiple levels. miRNAs are key regulatory factors during gene expression with the unique ability to modulate hundreds of target genes, thus making them ideal candidates to control the cellular process of complex tissue regeneration. Previously, we isolated total RNA, enriched for small microRNAs (less than 200 nucleotides) and performed deep sequencing for small RNAs.

In Year 2, we completed our analysis of miRNA expression for regenerating axolotl forelimbs and *Polypterus* pectoral fins. We identified 26/83 and 26/95 miRNAs that were significantly upregulated by at least 2-fold between the uninjured and regenerating axolotl and *Polypterus* samples respectively. Employing similar biostatistical parameters, we identified 33-shared miRNAs that are highly downregulated during active

appendage/limb regeneration. From this dataset, we selected the eight most highly differentially expressed, shared miRNAs for future studies (Figure 2). Surprisingly, the mature sequences of these miRNAs are highly conserved, despite these animals having diverged ~800 million years ago in evolution. In fact, at most, the mature miRNA sequence differs only by 2-nucleotides.

```
ame-miR-21        5′  UAGCUUAUCAGACUGAUGUUGAC  3′
pse-miR-21        5′  UAGCUUAUCAGACUGGUGUUGGC  3′

ame-miR-181a      5′  AACAUUCAACGCUGUCGGUGAGU  3′
pse-miR-181a      5′  AACAUUCAACGCUGUCGGUGAGU  3′

ame-miR-7a        5′  UGGAAGACUAGUGAUUUUGUUGUU  3′
pse-miR-7a        5′  UGGAAGACUAGUGAUUUUGUUGUU  3′

ame-let-7         5′  UGAGGUAGUUGUUUGUACAGUU  3′
pse-let-7         5′  UGAGGUAGUUGUUUGUACAGUU  3′

ame-miR-130c      5′  CAGUGCAAUAUUAAAAGGGCAU  3′
pse-miR-130c      5′  CAGUGCAAUAUUAAAAGGGCAU  3′

ame-miR-338       5′  UCCAGCAUCAGUGAUUUUGUUGA  3′
pse-miR-338       5′  UCCAGCAUCAGUGAUUUUGUUG  3′

ame-miR-204       5′  UUCCCUUUGUCAUCCUAUGCCU  3′
pse-miR-204       5′  UUCCCUUUGUCAUCCUAUGCCU  3′

ame-miR-2184      5′  AACAGUAAGAGUUAAUGUGCUG  3′
pse-miR-2184      5′  AACAGUAAGAGUUUAUGUGUUG  3′
```

Figure 2. Alignment of mature miRNA sequence between axolotl (ame) and *Polypterus* (pse). Red nucleotides represent conserved sequences between the two different animal model systems.

Following our analysis of the deep-sequencing dataset, we proceeded to validate the expression changes with real-time qPCR studies. We confirmed that miR-21 is the most highly upregulated miRNA during both axolotl and *Polypterus* limb regeneration. Conversely, miR-181a, miR-7a, let-7, miR-130c, miR-338 and miR-204 were all significantly depleted during regeneration, when compared to uninjured levels. Interestingly, miR-2184 exhibited opposite expression changes (Figure 3). Whereas expression levels decrease in axolotl limbs, miR-2184 is robustly elevated during *Polypterus* appendage regeneration. From this subset of miRNAs, we have chosen miR-21 and miR-2184 for further in-depth functional studies during regeneration due to the strong expression changes in response to injury.

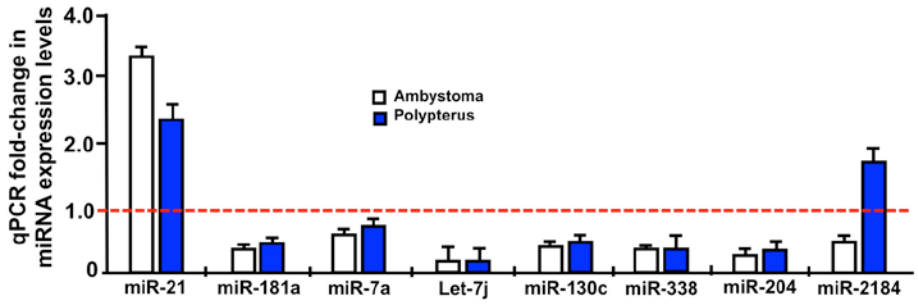

Figure 3. qPCR validation of shared miRNAs. Total RNA was isolated from uninjured and 7 dpa regenerating axolotl and *Polypterus* limbs for cDNA synthesis and qPCR analysis. miR-21 was strongly upregulated while miR-181a, miR-7a, let-7, miR-130c, miR-338 and miR-204 were reduced in expression during regeneration. miR-2184 was elevated in *Polypterus* but decreased in expression in axolotl samples. All levels are expressed as fold-change over the uninjured expression levels.

Specific Aim 3: Determine a genetic positional memory code for appendage regeneration. This work will be accomplished by **a**) isolating tissue and RNA from uninjured and regenerating zebrafish caudal fins, **b**) profiling miRNAs with microarray hybridization technology and **c**) filter and categorize the dataset into increasing and decreasing miRNA expression.

Research Accomplishments: To initiate our studies on positional memory, we isolated uninjured and regenerating tissue from specified regions of the zebrafish caudal fin. The amputation planes were designated proximodistal (PD) 1-3 (Figure 4). We collected tissue that was ~2 bony segments directly proximal to the PD amputation plane, isolated total RNA and performed profiling studies of miRNA expression at all planes of amputation in uninjured and regenerating samples.

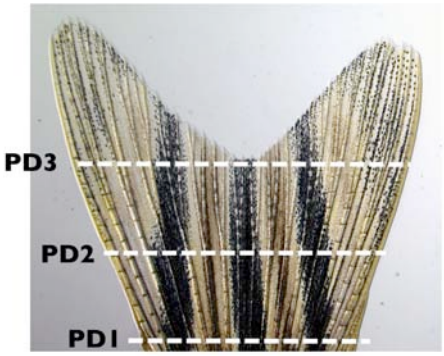

Figure 4. Schematic of positional memory amputation planes. Adult zebrafish caudal fins were independently amputated at proximodistal 1-3 (PD1-3). A second amputation plane, located at ~ 2 bony segments proximal to the original cut sites, were harvested for total RNA isolation.

From our profiling studies, we identified a subset of miRNAs that is differentially expressed at specific planes when comparing the uninjured samples to the regenerating groups (Table 2). Interestingly, comparisons between uninjured and regenerating tissues at PD1-PD3 revealed miR-21 and miR-181a are highly upregulated while miR-101 was consistent reduced in expression. This suggests, that these miRNAs could have similar roles in the regeneration process, regardless of the plane of injury.

Specific Aim 4: Define requirements for region-specific regulatory factors in maintaining positional memory. This work will be accomplished by **a**) validating miRNA expression using Northern blot hybridization and/or real-time quantitative PCR, **b**) determining spatial resolution of miRNAs during regenerative states and **c**) performing functional studies on miRNAs using antisense oligonucleotides to determine the effects on regeneration.

Table 2. Differentially expressed miRNAs during appendage regeneration.

UPD1 vs RPD1 Upregulated	UPD1 vs RPD1 Downregulated	UPD2 vs RPD2 Upregulated	UPD2 vs RPD2 Downregulated	UPD3 vs RPD3 Upregulated	UPD3 vs RPD3 Downregulated
dre-miR-21	dre-miR-738	dre-miR-21	dre-miR-101a	dre-miR-21	dre-miR-139
dre-miR-181a*	dre-miR-2190	dre-miR-181a*	dre-miR-2184	dre-miR-181a*	dre-miR-2190
dre-miR-451	dre-miR-204	dre-let-7i	dre-miR-204	dre-miR-15c	dre-miR-738
dre-miR-2188	dre-miR-101a	dre-miR-181b	dre-miR-338	dre-miR-15b	dre-miR-101a
dre-miR-31	dre-miR-2184	dre-miR-15c	dre-miR-29b	dre-miR-140*	dre-miR-338

Our initial experimental design to examine genetic indicators of positional memory enabled us to not only examine differentially expressed miRNAs at the individual amputation planes, but also allowed us to identify miRNAs that are expressed in a gradient from PD1-PD3 (Figure 4). To validate changes in expression levels, we used real-time qPCR studies. Consistent with the deep-sequencing dataset, miR-21 and miR-181a exhibited robust upregulation when appendages are challenged with injury. In contrast, miR-101 and miR-2184 are highly depleted with the onset of injury (Figure 5). While Figure 5 depict results from studies conducted at PD2, all four-candidate miRNAs exhibited similar expression changes at all three amputation planes, when compared to the uninjured tissue. For future studies, we selected miR-21 and miR-101 because they represent the most highly upregulated and depleted miRNAs from our deep-sequencing dataset.

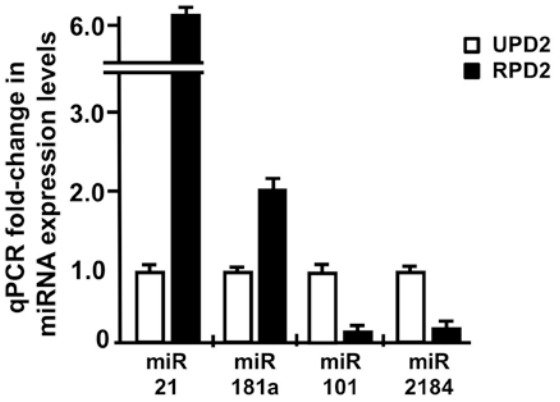

Figure 5. Real-time qPCR studies validate the change in miRNA expression. Levels of miR-21 and miR-181a are highly upregulated during zebrafish appendage regeneration in response to injury. Conversely, miR-101 and miR-2184 are strongly reduced in expression upon amputation.

Additionally, we filtered the entire dataset of small miRNAs from uninjured and regenerating tissue samples in order to identify miRNAs that are expressed in a gradient and differentially regulated between uninjured and regenerating states. From this sub-group of potential genetic indicators of positional memory, we chose miR-196c, miR-20a*, miR-738 and miR-218b for additional studies (Figure 6). While miR-196c has been implicated in limb development and patterning, the contributions of the remaining three miRNAs have not been investigate during limb development or regeneration. Interestingly, bioinformatics queries identified components of the retinoic acid pathway, a key pathway for

positional identity, as putative target genes for miR-20a*, miR-738 and miR-218b. These miRNAs would represent the first known posttranscriptional regulators of RA signaling.

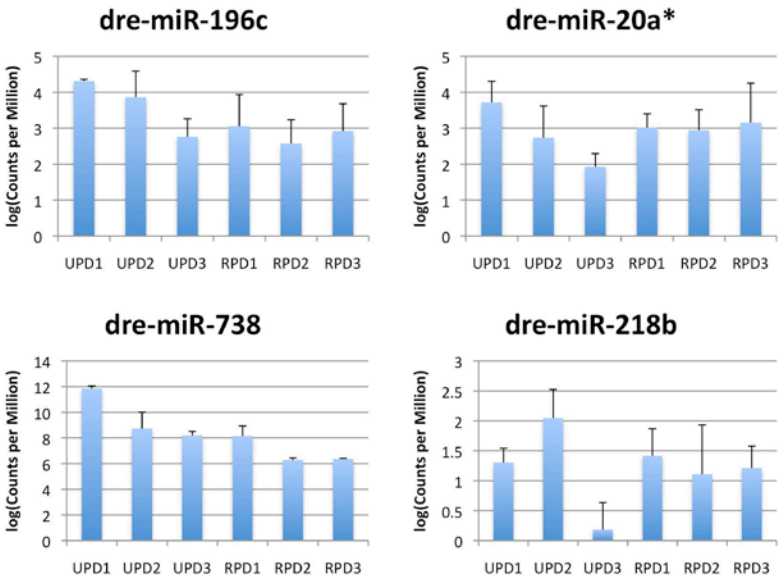

Figure 6. Gradient miRNA expression in uninjured and regenerative limb tissues. Expression levels of miR-196C, miR-20*, miR-738 and miR-218b are depicted as log values of reads per million total sequence tags. All four miRNAs also exhibit significant changes in expression between uninjured and regenerating tissues at all amputation planes.

Specific Aim 5: Grow Mount Desert Island Biological Laboratory as a collaborative center for the Regenerative Biology and Medicine research community. This work will be accomplished by establishing a MDIBL Visiting Scholars Program in Regenerative Biology.

Research Accomplishments: Our advertisement for Fellowships in Regenerative Biology and Medicine was sent to domestic and international research labs studying regeneration and to department heads at academic medical centers. From the collection of highly competitive applications, the selection committee reviewed and awarded three Regeneration Fellowships to Dr. Robert Morris from the Wheaton College, Dr. Kenneth Poss from Duke Medical Center and Dr. Jorge Contreras from University of New Jersey School of Medicine and Dentistry. These investigators are scheduled to deliver a scientific seminar during their time at MDIBL.

KEY RESEARCH ACCOMPLISHMENTS:

- Initiated functional studies to examine the roles of ECM components during cellular reprogramming in response to injury and regeneration.
- Successfully analyzed the small RNA deep-sequencing dataset to complement mRNA transcriptome dataset in amphibian studies.
- Identified shared miRNAs that are activated in response to axolotl, *Polypterus* and zebrafish limb regeneration.
- Completed the analysis of deep-sequencing studies on small noncoding RNAs to identify potential genetic regulators of positional memory during appendage regeneration.
- Successfully recruited and awarded regeneration fellowships to prominent regeneration scientists.

REPORTABLE OUTCOMES:

Poster presentation and abstract at the 3rd North Atlantic Zebrafish Research Symposium:

miR-21 is an evolutionarily conserved regeneration miRNA

Heather Carlisle, Ashley Smith, Benjamin King and Viravuth P. Yin

Davis Center for Regenerative Biology and Medicine, Mount Desert Island Biological Labs, Salisbury Cove, ME 04672, USA

ABSTRACT

Appendage regeneration is defined by the transformation of quiescent, differentiated tissues into highly proliferative and regenerative blastemal cells. These dramatic cellular changes are accompanied with rapid

modulation of gene expression, thus implicating miRNAs. Here we performed deep-sequencing studies to identify shared regeneration miRNAs among zebrafish caudal fins, axolotl forelimbs and *Polypterus* pectoral limbs. Real-time Q-PCR analysis confirmed the evolutionarily conserved miR-21 is one of the most highly upregulated miRNAs in response to injury. *In situ* hybridization studies in zebrafish caudal fins reveal miR-21 expression is localized to the basal-epithelial tissue layer and distal blastemal cells. Experimental depletion of miR-21 levels with antisense oligonucleotides culminated in regenerative outgrowth and patterning defects in all three animal systems. Furthermore, we show in the zebrafish that miR-21 is essential to activate blastema formation and cell proliferation. Using an integrated bioinformatics approach, we identified *bmp3, timp3* and *fgf20a* as miR-21 putative target genes. Collectively, our studies implicate miR-21 as a key component of a miRNA genetic circuit for repair and regeneration of complex appendage tissues.

This project was supported by grants from the National Center for Research Resources (5P20RR016463) and the National Institute of General Medical Sciences (8 P20 GM103423) from the National Institutes of Health (V.P.Y) and from TARTC and USAMRAA (W81XWH-11-1-0425 (V.P.Y).

CONCLUSIONS:

In order to develop potential therapies to restore and/or augment human limb regeneration, we must first understand the molecular regulation of appendage regeneration in vertebrates that have retained enhanced regenerative capacity during evolution. Two critical limitations have impeded progress in this area: 1) lack of diverse experimental animals has precluded the powerful comparative approaches that have vertically advanced other fields of regenerative medicine such as stem cell biology; 2) unbiased functional genomic approaches have not been fully exploited. The experimental design we advance in this proposal integrates two innovative approaches to address these issues, and distinguishes this work as a unique and complementary extension of current projects in TATRC's portfolio. We have and will continue to use a novel comparative approach employing phylogenetically diverse experimental organisms, and adopt unbiased systems-level approaches to identify and dissect the gene networks initiating and maintaining regenerative responses in vertebrate limbs.

In Year 2, we have achieved all milestones outlined in the original research proposal. In brief, a major finding that emerged from our small RNA deep-sequencing studies of *Polypterus,* axolotl and zebrafish limb regeneration studies is the identification of a core group of differentially expressed, shared miRNAs. Given the ability of miRNAs to control gene expression of entire genetic programs, we believe miR-21, miR-181a and miR-101 constitute a core regeneration network that is involved in the natural reprogramming of differentiated cells. In preliminary studies, we have identified many components of the ECM as potential target genes of miR-21. It is our developing hypothesis that miR-21/ECM pathway is important in coordinating not only cellular movement but also is vital for cellular dedifferentiation during the initiation of the regenerative response. Therefore, we will direct our future functional studies to examine this genetic circuit. It is our expectation that our recent progress and the collective aims of this proposal will provide novel fundamental insight into the molecular control of appendage regeneration, vertically advancing our understanding of the mechanistic causes of regenerative failure in mammals, and ultimately guiding the targeted development of *in vivo* therapies to restore/augment human limb regeneration.

REFERENCES: N/A

APPENDICES: N/A

SUPPORTING DATA: N/A